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Stable chelating linkage for reversible immobilization of oligohistidine tagged proteins in the BIAcore surface plasmon resonance detector

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Abstract

We describe a stable chelating linkage for the reversible immobilization of oligohistidine tagged proteins in the flow cell of the 'BIAcore' surface plasmon resonance (SPR) biosensor. The carboxymethylated dextran surface of the flow cell was covalently derivatized with *N*-(5-amino-1-carboxypentyl)iminodiacetic acid (NTA ligand) via its single primary amino group, and the derivatized surface charged with Ni^{2+}. 6His-VP55, an N-terminally tagged derivative of the catalytic subunit of the heterodimeric vaccinia virus poly(A) polymerase, was immobilized to this surface in a manner that was dependent upon the immobilized NTA ligand, the prior injection of Ni^{2+} at a concentration of $> 10^{-5}$ M and the 6His tag, and which was reversible upon injection of EDTA. The stability of immobilization varied inversely with the amount of 6His-VP55 immobilized and was greatest in buffer of pH 8.0 or greater, containing NaCl at a concentration of 0.1 M. Utilizing these conditions, 6His-VP55 remained stably immobilized during 60 min of buffer flow at moderate flow rates. VP39, the stimulatory subunit of vaccinia poly(A) polymerase, interacted with the immobilized 6His-VP55. ~ 99% of immobilized 6His-VP55 molecules were available for VP39 binding, in contrast to the ~ 40% availability for 6His-VP55 molecules immobilized covalently, via primary amino groups. Three additional proteins, tagged at either the N- or C-terminus with oligohistidine, were shown to be stably immobilized via the chelating linkage. This simple method permits immobilization of proteins in the BIAcore biosensor via a commonly employed affinity tag, in a stable and reversible manner, and requires only a single biosensor flow cell for the iterative generation of immobilized protein surfaces.

Keywords: BIAcore; Histidine; Nickel; Metal chelate; Transition metal complex

Abbreviations: EDC, *N*-ethyl-*N*'-(3-diethylaminopropyl)-carbodiimide; IMAC, immobilized metal ion affinity chromatography; NHS, *N*-hydroxysuccinimide; NTA [nitrilotriacetic acid] ligand, *N*-(5-amino-1-carboxypentyl)iminodiacetic acid; RU, resonance units; SPR, surface plasmon resonance.

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1. Introduction

SPR is an increasingly popular technology for the analysis of biomolecular interactions in real-time (Jönsson et al., 1991). In the BIAcore SPR biosensor made by Pharmacia, one of a pair of interacting proteins must be immobilized within the biosensor flow cell before monitoring the interaction of the mobile-phase partner. A number of strategies have been devised for protein immobilization. In one common strategy, the protein is covalently immobilized via its superficial primary amino groups (Johnsson et al., 1991). However, this method suffers from the potential drawbacks of both creating a heterogeneous surface, and coupling via surfaces of the protein required for other interactions to be studied in the biosensor. In addition, an absence of basic residues in the protein would obviate the method entirely. Greater selectivity in the point of protein attachment has been obtained by immobilization via less frequently occurring moieties, such as superficial reactive thiols (O'Shannessy et al., 1992). In one elegant variation to this approach, selective immobilization was achieved via a single thiol engineered into a recombinant protein (Cunningham and Wells, 1993). However, this variation would require that reactive thiols are not already present in the protein. For still greater specificity in protein attachment, a high-affinity ligand such as biotin, avidin, streptavidin, IgG with oxidized sugars or the Fc portion of anti-mouse IgG can be covalently immobilized in a group-specific manner, followed by the non-covalent immobilization of the target protein or a monoclonal antibody thereto, via a specific high-affinity interaction (Dubs et al., 1991; O'Shannessy et al., 1992). However, this methodology may require different high-affinity ligands for the immobilization of different species or classes of protein. There is a requirement for methods which can immobilize proteins via a specific domain that occurs once within numerous different proteins and does not interact with domains required for association with other molecules, and which employ a regeneratable linkage. Expression of recombinant proteins tagged at their termini with oligohistidine is a popular strategy for their rapid single step purification by immobilized metal ion affinity chromatography (IMAC) (Porath et al., 1975; Porath and Olin, 1983; Hochuli et al., 1987; Janknecht et al., 1991). Here, we describe the stable, reproducible and reversible immobilization of such proteins in the BIAcore biosensor flow cell, via a chelating linkage. This simple method permits protein immobilization in the BIAcore biosensor via a commonly employed affinity tag, in a stable and reversible manner, and requires only a single biosensor flow cell for the iterative generation of immobilized protein surfaces.

2. Materials and methods

2.1 Reagents

NTA ligand was a kind gift of Joachim Ribbe, Diagen, Germany. It was also synthesized by the authors, following the method described by Hochuli et al. (1987). Disodium EDTA was obtained from GIBCO-BRL as a 0.5 M stock solution (pH 8.0). Tween 20 was purchased either from Pharmacia Biosensor as a 10% solution named 'surfactant P20', or from Pierce. *N*-ethyl-*N'*-(3-diethylaminopropyl)-carbodiimide (EDC), *N*-hydroxysuccinimide (NHS) and ethanolamine were purchased as part of the 'amine-coupling kit' (Pharmacia Biosensor). Human La protein, tagged with 6 histidines at its C-terminus, was kindly provided by Fiona Topfer after its over-expression in *E. coli* and purification to apparent homogeneity using chelating agarose. Thrombin protease was obtained from Novagen.

2.2. Proteins

VP55, N-terminally tagged with six consecutive histidine residues and a thrombin cleavage site (6His-VP55), was expressed in *E. coli* using the expression vector pET15b (Novagen), then purified to apparent homogeneity from an *E. coli* extract as detailed separately (P.D. Gershon, in preparation). In the final purification step, 6His-VP55 was applied to Ni^{2+}-NTA-agarose (Qiagen), and eluted with an ascending gradient of imidazole. 6His-VP55 was concentrated for covalent coupling to the BIAcore flow cell surface by

vacuum dialysis of Ni^{2+}-NTA-agarose column fractions, with complete buffer exchange against 2% glycerol, 5 mM Hepes-NaOH (pH 7.9), 2 mM 2-mercaptoethanol. 6His-VP55 was concentrated for immobilization by chelation, by ultrafiltration of Ni^{2+}-NTA-agarose column fractions using a Centriprep 10 (Amicon), with partial buffer exchange, so that the resulting preparation contained 4% glycerol, 12 mM Hepes-NaOH (pH 7.9), 1.3 mM 2-mercaptoethanol, 65 mM NaCl, 25 mM imidazole. 6His-VP55 was cleaved with thrombin after concentration from Ni^{2+}-NTA-agarose column fractions using a Resource S column (Pharmacia) run in 5% glycerol, 10 mM Hepes-NaOH (pH 7.9), 2 mM 2-mercaptoethanol, eluting the protein with a steep ascending gradient of NaCl. Gradient fractions were made 2.5 mM in $CaCl_2$ and incubated with 20 U of thrombin protease per mg of 6His-VP55, for 2 h at 23° C. The completeness of digestion was confirmed by SDS PAGE. For use as a liquid-phase 'analyte' the digestion mixture was passed through a Bio-Spin 6 column (Bio-Rad) following the manufacturer's instructions, with buffer exchange against buffer A (10 mM Hepes-NaOH pH 7.0, 150 mM NaCl, 0.005% Tween 20, in H_2O of resistance > 16 MΩ/cm at 25° C).

N-terminally 6His-tagged VP39, with a protease factor Xa site for tag removal (6His-VP39) and C-terminally 6His-tagged VP39 were expressed in *E. coli* as detailed separately (P.D. Gershon, in preparation), and purified to apparent homogeneity in a single step using Ni^{2+}-NTA-agarose as described above for VP55. The over-expression and purification of wild-type VP39 from vaccinia virus-infected cells has been described (Gershon and Moss, 1993).

2.3. SPR

SPR experiments were performed using the BIAcore integrated device (Pharmacia Biosensor), with which binding data were recorded in real time as 'sensorgrams'. Running buffer for all experiments was buffer A (above). The carboxymethylated dextran surface of the biosensor chip CM-5 was derivatized with NTA ligand by activation with EDC (0.05 M) and NHS (0.2 M) for 10 min, followed by injection of NTA ligand (50 mM in H_2O, pH 8.0, 10 min). Remaining NHS groups were blocked by injection of 1 M ethanolamine (2.5 min). Oligohistidine-tagged proteins were immobilized by injection of Ni^{2+} solution (2–5 min) followed by the tagged protein diluted in buffer A. Tagged proteins and residual interacting ('analyte') proteins were eluted by injection of EDTA (0.5 M, pH 8.0). The duration of injections, composition of Ni^{2+} solution and flow rates varied in individual experiments, as indicated in figure legends. 6His-VP55 was covalently immobilized to the sensor chip surface via its primary amino groups following activation of the carboxymethylated dextran layer with EDC and NHS as for covalent immobilization of NTA ligand (above), except that a 97 sec injection of 6His-VP55 (20 nM in 20 mM sodium acetate pH 5.0) replaced the injection of NTA ligand. VP39 was diluted in buffer A prior to its injection as 'analyte'.

2.4. Determination of kinetic rate constants

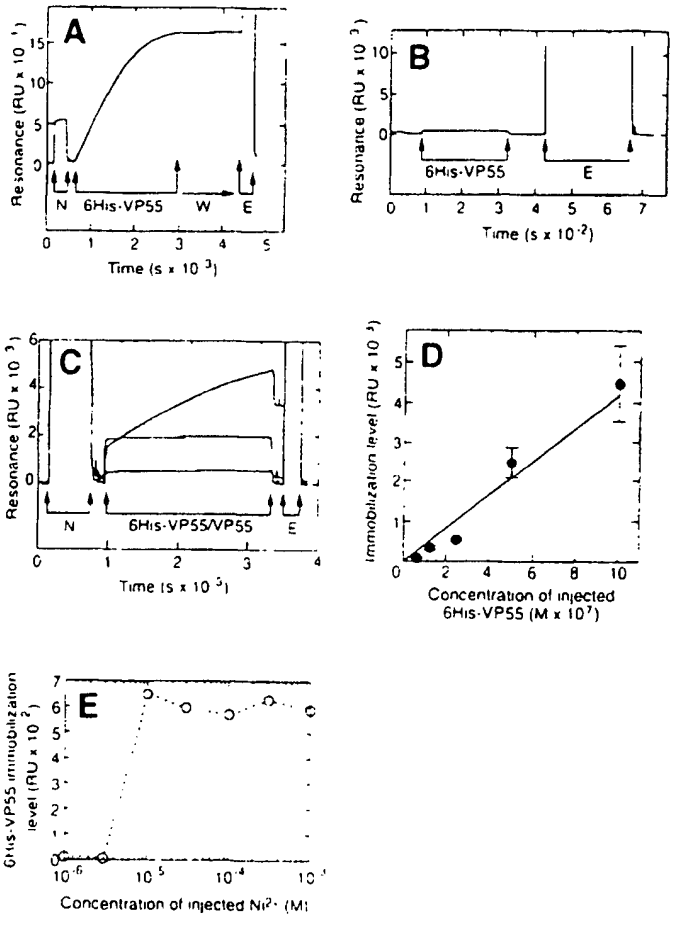
Text files containing sensorgram data were transferred from the BIAcore to a Macintosh IIfx computer, and curve fitting performed using the IGOR Pro Graphing and Data Analysis software (WaveMetrics, Lake Oswego, OR) as originally suggested by O'Shannessy et al. (1993). For dissociation rate constant determination, the ordinates of sensorgrams were adjusted, to set the resonance signal immediately before the start of protein injection to zero, and the abscissa was adjusted to set time zero to 1 min after the start of buffer washout. Dissociation phases of the sensorgrams were fitted to the exponential function $B_t = B_0 \exp(-k_{diss} \cdot t)$, where B_t = resonance signal in resonance units (RU) at time t (s), B_0 = resonance signal (RU) at time 0, and k_{diss} is the dissociation rate constant (s^{-1}).

3. Results and discussion

3.1. Requirements for interaction of 6His-VP55 with the NTA-derivatized biosensor flow cell surface

NTA ligand was covalently immobilized via its single primary amino group at the BIAcore

biosensor flow cell surface, following surface activation with EDC + NHS as described above. Before injection of 6His-tagged protein, a metal charging step was included, to mimic the resin charging step included prior to IMAC of proteins (Porath et al., 1975; Porath and Olin, 1983; Hochuli et al., 1987) (Fig. 1A). In the majority of experiments charging was performed in the presence of excess Tris-HCl (pH 7.5), to buffer the $NiSO_4$ and also possibly present the Ni^{2+} as a complex with Tris. The catalytic subunit of vaccinia virus poly(A) polymerase, VP55 (Gershon and Moss, 1992), tagged at its N-terminus with six consecutive histidine residues (6His-VP55), was employed in initial immobilization experiments. 6His-VP55 interacted strongly with the Ni^{2+}-charged, NTA-derivatized surface, and 91% of the bound protein was eluted with a 10 min injection of 0.5 M EDTA (pH 8.0) (Fig. 1A). Routinely, ~99% of bound protein was eluted after a second EDTA injection. 6His-VP55 did not bind the NTA-derivatized surface in the ab-



sence of Ni^{2+} charging (Fig. 1B), and VP55 from which the 6His tag had been cleaved did not interact with Ni^{2+}-charged NTA-derivatized surfaces (Fig. 1C, middle trace). In addition, 6His-VP55 did not bind a mock-derivatized flow cell in which the NTA ligand injection step had been omitted, after injection of Ni^{2+} (Fig. 1C, lower trace). These experiments showed that 6His-VP55 could be immobilized at the biosensor flow cell surface in a manner that is dependent upon surface-bound NTA ligand, Ni^{2+} and an oligohistidine tag on the protein, and that is reversible upon injection of EDTA.

Approximately 16000 RU of 6His-VP55 was immobilized in Fig. 1A. In other experiments, immobilization levels were routinely found to plateau after the binding of 16000–18000 RU of 6His-VP55, (data not shown), indicating the saturation of chelating surfaces. 18000 RU corresponds to a surface concentration of immobilized protein of approximately 3.2 mM, assuming a dextran layer of 100 nm thickness, and a 1000 RU rise in resonance in response to 1 ng/mm^2 of bound protein (Stenberg et al., 1991). On injection of various sub-saturating concentrations of 6His-VP55 to the NTA-derivatized flow cell in binding-elution cycles of the form shown in Fig. 1A, levels of protein immobilized were directly proportional to the concentration of protein injected (Fig. 1D). In the above experiments, the injected Ni^{2+} was at a concentration of 100 mM or 250 mM. In later experiments (Fig. 1E), reduction of Ni^{2+} concentrations to levels as low as 10 μM, in 2–4 min injections, was found not to affect the level of 6His-VP55 immobilized. On reduction of Ni^{2+} concentrations to below 10 μM, both 6His-VP55 binding and the signal normally ascribed to immobilized Ni^{2+} dropped equally dramatically (Fig. 1E, data not shown), indicating that the primary effect of low Ni^{2+}

Fig. 1. Chelating linkage for immobilization of 6His-VP55 in the SPR biosensor. *A*: sensorgram showing the immobilization of 6His-VP55 in an NTA-derivatized flow cell, and its elution with EDTA. Injection periods are indicated with vertical arrows: 'N' = $NiSO_4$ (0.25 M, 5 min); '6His-VP55' = 6His-VP55 (2 μM in buffer A, 40 min, leading to a 16128 RU increase in resonance); 'E' = EDTA (0.5 M, pH 8.0, 5 min); 'W' = washout of flow cell with buffer A (21 min). The running buffer flow rate was 1 μl/min. The ordinate was adjusted to set the resonance value immediately before the 'N' injection to zero. *B*: sensorgram showing the Ni^{2+} dependence of immobilization. After EDTA treatment of the flow cell used in Fig. 1A, 6His-VP55 injection (1 μM, 4 min, giving a 1 RU increase in resonance), was followed by EDTA injection (4 min). The running buffer flow rate was 10 μl/min. Other symbols and conditions are as for *A*. *C*: dependence of the chelating linkage on an NTA ligand-derivatized sensor chip surface and the presence of a 6His tag. 'N' = 10 min injection of $NiSO_4$ (0.1 M)/Tris-HCl (0.3 M, pH 7.5); 'E' = EDTA (0.5 M, pH 8.0, 4 min, 10 μl/min). Other symbols and conditions are as for *A*. *Upper trace*: control sensorgram. 6His-VP55 (0.4 mM) was prepared for thrombin cleavage as described (materials and methods section), and 50% of this material was injected to a Ni^{2+}-charged, NTA-derivatized flow cell surface, leading to a 3164 RU rise in resonance. *Middle trace*: proteolytic removal of the 6His tag prevents VP55 binding to the chelating surface. After incubating the remaining 6His-VP55 mixture used for the upper trace with thrombin (materials and methods section), the injection cycle was repeated. Injection of the cleavage mixture was accompanied by a 50 RU rise in resonance, corresponding to the mass of the isolated 6His tag. *Lower trace*: the chelating linkage requires NTA ligand derivatization of the flow cell surface. 6His-VP55 (2 μM) was injected to a mock-derivatized flow cell surface (NTA ligand injection step was omitted during derivatization) which was accompanied by a negligible rise in resonance. *D*: linear relationship between concentration of injected 6His-VP55 and immobilization level. Ten cycles similar to that shown in *A* were performed, employing five concentrations of 6His-VP55 for injection (10 min), and duplicate cycles at each concentration. Ni^{2+} charging comprised a 2.5 min injection of $NiSO_4$ (0.25 M)/Tris-HCl (0.3 M, pH 7.5). The washout duration was 20 min. EDTA injection duration was 8 min and the running buffer flow rate was 4 μl/min. 'Immobilization level' (ordinate) = difference between resonance signals immediately before and after 6His-VP55 injection. Points indicate mean values and error bars show range. A linear regression of the mean values is shown. *E*: concentrations of $Ni^{2+} \geq 10^{-5}$ M are sufficient for charging of the chelating surface. Replicate cycles of the form shown in *B* were performed, except that the flow rate throughout the experiment was 10 μl/min, and the charging step comprised a 4 min injection of serially diluted $NiSO_4$ (0.1 M)/Tris-HCl (0.3 M, pH 7.5) in H_2O. The net rise in resonance over each 4 min injection of 6His-VP55 is plotted against Ni^{2+} concentration. Charging with Ni^{2+} at concentrations between 1 and 250 mM (data not shown) led to 6His-VP55 immobilization levels equivalent to those obtained at Ni^{2+} concentrations of 10^{-2} M–10^{-1} M (plotted).

concentration might be abrogation of the Ni^{2+}-NTA interaction. A Ni^{2+} concentration of 1 mM is considered adequate for practical purposes.

3.2. Factors affecting stability of the chelating linkage

The stable immobilization of tagged proteins during prolonged buffer flow is very important for quantitative analysis of their interactions with mobile-phase molecules. Thus, factors affecting immobilization stability were examined, starting with levels of injected protein. In each of the binding-regeneration cycles conducted for Fig. 1D, buffer A was directed to the flow cell for 20 min after the completion of 6His-VP55 injection, and resonance was monitored. The stability of 6His-VP55 immobilization was quantified from the drop in resonance over this 20 min washout period. 6His-VP55 elution occurred with apparent first order dissociation kinetics, permitting apparent rate constants for 6His-VP55 dissociation from the chelating surface (k_{diss}) to be determined by exponential curve fitting to the 'washout' portions of sensorgrams, as described in the materials and methods section. It is apparent (Fig. 2A), that increased levels of immobilized 6His-VP55 correlate with an increased rate of 6His-VP55 dissociation from the chelating surface, i.e. a net decrease in the stability of immobilization. In other experiments, the apparent k_{diss} was also found to increase with washout buffer flow rate (data not shown). The effects of immobilization level and buffer flow rate indicate that oligohistidine-tagged protein transiently elutes from, and re-binds to the chelating surface, and that the instability observed at high levels of immobilized protein results from a reduced availability of free chelating groups to re-bind transiently eluted protein molecules.

The occurrence of transient elution and re-binding, consistent with the laws of mass action, would predict that, at any level of immobilized 6His-VP55, a net elution of immobilized protein would commence after the passage of sufficient running buffer through the flow cell. However, by employing appropriate immobilization levels (~1000 RU), and buffer flow rates (< 10 μl/min), immobilized 6His-VP55 surfaces with very high net stability were routinely obtained during prolonged periods of buffer flow, in numerous experiments. Fig. 2B shows a typical, highly stable immobilization of 6His-VP55, in which a net change of −1 RU was recorded over a 60 min washout period, with resonance fluctuations of no more than ±2 RU during this period. Although no attempt was made to maximize the surface density of covalently bound NTA ligand, our data would suggest this to be an additional means for increasing immobilization stability at elevated flow rates and levels of immobilized protein.

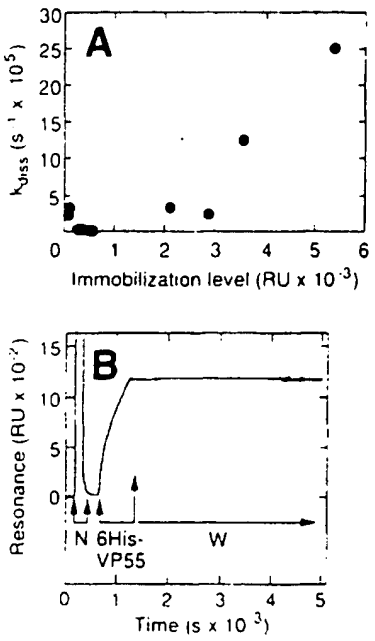


Fig. 2. Stability of 6His-VP55 immobilization in the chelating flow cell. *A*, dependence of apparent 6His-VP55 dissociation rate (k_{diss}) on the initial level of immobilization of 6His-VP55. Exponential functions were fit to the 'washout' portions of the sensorgrams from the experiment described under Fig. 1D, as described in the materials and methods section. Resulting k_{diss} values are plotted against the initial levels of 6His-VP55 immobilized. *B*, sensorgram showing the stable immobilization of 6His-VP55 in the chelating flow cell. 6His-VP55 injection (0.3 μM, 4 min) led to a 1149 RU increase in resonance. W = 60 min washout. Ni^{2+} charging and running buffer flow rate were as for Fig. 1D. Other symbols and conditions are as for Fig. 1A.

In the above experiments (Figs. 1 and 2), the washout buffer (buffer A) contained 150 mM NaCl and Hepes buffer at pH 7.0. However, for the immobilization scheme to have utility with other 6His-tagged proteins, the chelating linkage may need to be stable to other buffer conditions. Initially, we determined the effects of pH and salt concentration on linkage stability. Thus, 6His-VP55 was immobilized to the Ni^{2+}-charged NTA-derivatized surface, washed for 10 min with buffer A, and the resulting surface challenged with buffers of different pH and salt concentration for 11 min, before elution of the tagged protein with EDTA. A fresh binding-elution cycle was performed for each challenge buffer. From the data of Fig. 2, levels of injected 6His-VP55 were chosen that would lead to less than completely stable immobilization in buffer A. This was done to facilitate the detection of conditions that may lead to increased immobilization stability. Rate constants were calculated for 6His-VP55 dissociation during the challenge phases of the resulting sensorgrams, by exponential curve fitting. The resulting apparent k_{diss} values are shown in Table 1. As predicted, only a metastable surface (apparent $k_{diss} = 2.3 \times 10^{-3}$ s^{-1}) was obtained in buffer A. At 150 mM NaCl, the linkage was further destabilized by changing pH from 7.0 to 6.0, consistent with the use of low pH conditions for the elution of tagged proteins from IMAC columns (Porath et al., 1975). The linkage was stabilized by changing pH from 7.0 to 8.0 or above. A small reproducible increase in resonance was observed during the injection of pH 9.0 buffer (Table 1), which was independent of buffer species (data not shown). The cause of this increase is unknown, though one explanation might be a minor pH-dependent aggregation of VP55 molecules at the flow cell surface. At pH 7.0, the linkage was most stable at an NaCl concentration of approximately 100 mM. The linkage was reproducibly less stable at other NaCl concentrations, with the most significant destabilization occurring at NaCl concentrations greater than 150 mM. This experiment indicated that optimal stability was conferred in pH 8.0 buffer containing NaCl at a concentration of 100 mM, rather than under our standard conditions of pH 7.0 and 150 mM NaCl (buffer A). However, since perfectly stable surfaces could routinely be obtained in buffer A at moderate levels of immobilized oligohistidine-tagged protein (cf. Fig. 2A), the immobilization-level parameter could apparently be used to control stability under sub-optimal tonic conditions. The precise immobilization level, and levels of pH and salt concentration compatible with stability varied slightly with different NTA-derivatized surfaces, and with the

Table 1
Effects of pH and salt concentration on the chelating linkage

pH	NaCl conc. (mM)	Amount of 6His-VP55 initially immobilized (RU)	Apparent k_{diss} (s^{-1})
7	50	3689	4.0×10^{-5}
7	100	3487	7.9×10^{-6}
7	150	3642	2.3×10^{-3}
7	250	3263	2.2×10^{-4}
7	500	2574	3.6×10^{-4}
7	1000	3167	4.2×10^{-4}
6	150	3039	6.4×10^{-4}
8	150	2924	6.8×10^{-6}
9	150	2871	-6.4×10^{-5}

Nine cycles (shown on separate lines) each comprised charging (as in Fig. 1D), injection of 6His-VP55 (0.8 μM), a 10 min washout with buffer A, an 11 min injection of challenge buffer, and EDTA elution (as in Fig. 1D), at a flow rate of 4 μl/min. For pH 6.0 and 9.0 challenges, MES-NaOH and CHES-HCl buffers (20 mM), respectively, were used. Challenge buffers contained Tween 20 (0.005%). Surface stability in challenge buffer is inversely proportional to apparent k_{diss}, calculated as described in the materials and methods section. The negative value for apparent k_{diss} at pH 9.0 indicates a small net increase in resonance signal that consistently occurred during challenge at this pH (see text).

age of a particular surface. This was the only observed effect of NTA-derivatized flow cell aging. The stability of the chelating linkage was not affected detectably by the reducing agents dithiothreitol, 2-mercaptoethanol or Tris(2-carboxyethyl)phosphine) at moderate concentrations (< 10 mM, data not shown), but stability was severely impaired by challenge with EDTA or imidazole at concentrations as low as 1 mM (data not shown). The possible presence of these reagents in protein samples purified by IMAC emphasizes the necessity for buffer exchange before protein injection.

NTA ligand is based upon nitrilotriacetic acid, which is quadridentate for Ni^{2+} (Hochuli et al., 1987). Other, commercially available ligands, such as iminodiacetic acid (IDA) were not tried. However, the lower Ni^{2+} binding stability of IDA with respect to NTA ligand (Hochuli et al., 1987), presumably due to its tridentate nature, may be limiting in the overall stability of the chelating linkage. Interestingly, the sensitivity of the NTA-Ni^{2+}-6His linkage to destabilization by NaCl and imidazole appears to be greater in the BIAcore than in IMAC format, and the sensitivity to strong reducing agents such as dithiothreitol, appears lower. The BIAcore format may be useful for the investigation of putative protein sequences capable of more stable metal co-ordination, and for more general studies of transition metal complexes with biopolymers.

3.3. Interaction of VP39 with non-covalently immobilized 6His-VP55: Kinetics and stoichiometry

VP39, the stimulatory subunit of vaccinia virus poly(A) polymerase is known to interact with VP55, the catalytic subunit (Gershon et al., 1991; Gershon and Moss, 1993) with a stoichiometry of 1 (Moss et al., 1975). After stable immobilization of 1131 RU of 6His-VP55 using the chelating

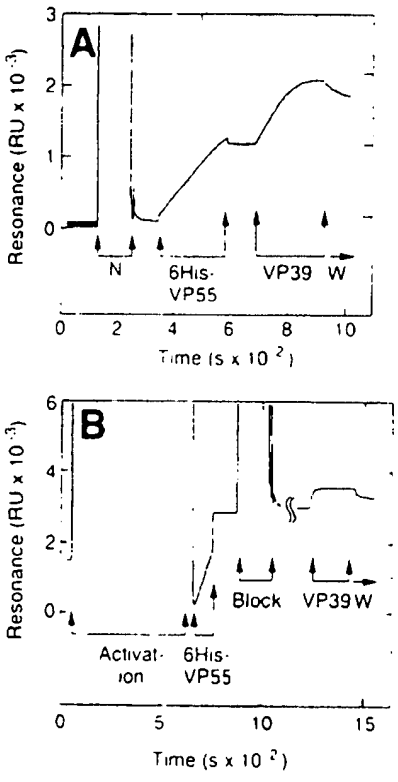


Fig. 3. Comparison of chelating with covalent linkage. *A*: interaction of soluble VP39 with 6His-VP55 immobilized via a chelating linkage. The sensorgram shows the immobilization of 6His-VP55 by chelation, and subsequent interaction of VP39 (100 nM). The fast changes in resonance at the start and end of VP39 injection, due to glycerol in the VP39 buffer, have been subtracted. The running buffer flow rate for the washout period after VP39 injection was 100 μl/min, at all other times it was 10 μl/min. 'N' = injection of 0.1 M $NiSO_4$ in 0.3 M Tris-HCl, pH 7.5 (2 min). '6His-VP55' = injection of 6His-VP55 (2.5 μM, 4 min – relatively low immobilization level due to elevated flow rate). 'VP39' = injection of VP39 diluted in buffer A (4 min). The cycle ended with an EDTA injection (8 min, not shown). Other symbols and conditions are as for Fig. 1A. In this experiment, 840 RU of VP39 were bound by 1189 RU of immobilized 6His-VP55. *B*: interaction of soluble VP39 with 6His-VP55 that was covalently immobilized via its primary amino groups. Immobilization (the 'activation', '6His-VP55', and 'block' steps on the figure), was performed as described in the materials and methods section. The sudden drop and rise in resonance at the start and end, respectively, of 6His-VP55 immobilization, reflects buffer change. VP39 (100 nM) binding, and washout (taken from a consecutive sensorgram), was performed as in *A*.

linkage, highly purified, vaccinia-expressed wild-type VP39 (Gershon and Moss, 1993) was injected into the flow cell followed by washout with buffer A, then EDTA elution of all bound protein. Fig. 3A shows the association of VP39 with, and its dissociation from the immobilized 6His-VP55 surface. Only very small amounts of VP39 interacted with the Ni^{2+}-charged surface in the absence of 6His-VP55 (data not shown), and this material dissociated rapidly (apparent $k_{diss} > 10^{-2}$ s^{-1}). The observed binding of VP39 was therefore specific for immobilized 6His-VP55. Control cycles were performed after covalently immobilizing a similar amount (1503 RU) of 6His-VP55, via its amino groups, in an adjacent flow cell of the biosensor chip (Fig. 3B). VP39 was injected to this 6His-VP55 surface, at the same concentration used in Fig. 3A, followed by washout with buffer A (Fig. 3B). As an index of the proportion of immobilized 6His-VP55 molecules available for VP39 interaction, the ratio of level of VP39 bound at saturation/6His-VP55 immobilized was calculated for the two surfaces, and the resulting value normalized for the respective molecular weights of VP55 and VP39. These calculations indicated that 99.6% of VP55 molecules immobilized by chelation were available for VP39 binding, whereas a value of only 40.2% was obtained for covalently immobilized VP55 molecules. Consistent results were obtained in replicate experiments. It thus appears that VP55 molecules anchored in a presumably uniform manner, via an N-terminal oligohistidine tag, are presented in a form amenable to subsequent molecular interaction, whereas VP55 molecules immobilized covalently, via superficial amino groups are not. The increased availability of terminally anchored molecules presumably re-

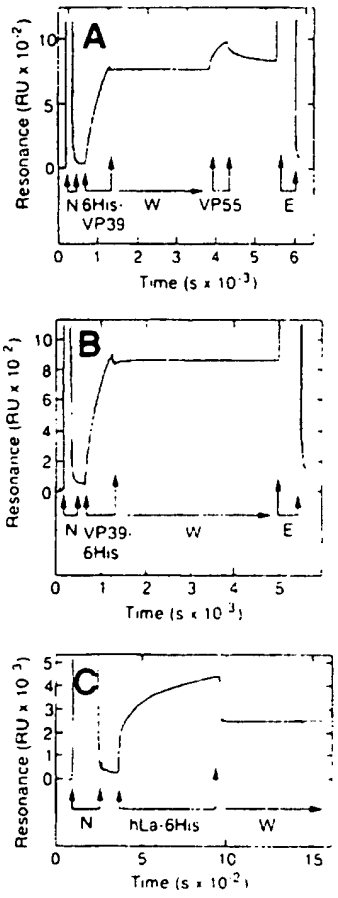


Fig. 4. Stable non-covalent immobilization of three additional 6His-tagged proteins in the chelating flow cell. *A*: immobilization of N-terminally 6His-tagged VP39 and its interaction with soluble VP55. '6His-VP39' = injection of 6His-VP39 (0.4 μM in buffer A, 10 min). The 'W' washout with buffer A (40 min), led to a net resonance change of +2 RU. 'VP55' = 8 min injection of thrombin-cleaved VP55 (0.2 μM in buffer A). Running buffer flow rate was 4 μl/min until the start of VP55 injection, after which it was 5 μl/min. Ni^{2+} charging and EDTA injection were as for Fig. 1D, other symbols and conditions are as for Fig. 1A. *B*: immobilization of C-terminally 6His-tagged VP39. 'VP39-6His' = injection of tagged VP39 (0.4 μM in buffer A, 10 min). The 'W' washout with buffer A (60 min) led to a net resonance change of +13 RU. The flow rate was 4 μl/min until the start of EDTA injection, after which it was 5 μl/min. All other symbols and details are as for part A. *C*: immobilization of C-terminally 6His-tagged human La protein. 'N' = injection of 0.25 M $NiSO_4$ (2 min). 'hLa-6His' = injection of the tagged La protein (1.5 μM in buffer A, 10 min). The large Δ resonance at the start and end of injection were due to glycerol and NaCl in the protein preparation. The 'W' washout with buffer A (11 min), led to a net resonance change of +23 RU. The flow rate was 4 μl/min. Other symbols and conditions are as for Fig. 1A.

sults from decreased steric hindrance, constraint upon molecular flexibility, and possible rate of inactivation. VP55 is a nucleic acid binding protein and, like other classes of nucleic acid binding and transcriptional regulatory proteins contains a high density of basic residues (Brendel and Karlin, 1989; Johnson and McKnight, 1989; Lazinski et al., 1989; Baxevanis and Vinson, 1993). This is consistent with its reduced availability for molecular interaction after the standard BIAcore covalent immobilization protocol.

To quantitate the interaction of VP39 with the two forms of immobilized 6His-VP55, apparent rate constants, k_{ass} and k_{diss} for the equilibrium:

$$\text{VP55} + \text{VP39} \underset{k_{diss}}{\overset{k_{ass}}{\rightleftharpoons}} \text{VP55} - \text{VP39}$$

were determined from replicate sensorgrams, including the data shown in Fig. 3, by non-linear curve-fitting (O'Shannessy et al., 1993; Corr et al., 1994). The accurate fitting of mono-exponential curves to portions of the association and dissociation phases of all sensorgrams confirmed apparent first-order kinetics. k_{ass} values of 5.3×10^5 and 2×10^5 M^{-1} s^{-1}, and k_{diss} values of 4×10^{-4} and 2.4×10^{-4} s^{-1}, were obtained for the covalent and non-covalent surfaces, respectively. The small differences between the two types of surface in apparent association and dissociation rate constant, were not considered significant.

3.4. Additional 6His-tagged proteins can be stably immobilized

The immobilization of three additional proteins was investigated, following the parameters for maximal stability outlined above. The first of these, *E. coli*-expressed N-terminally 6His-tagged VP39, was immobilized in a highly stable manner (Fig. 4A). When VP55 was injected into the flow cell, after proteolytic removal of the 6His tag from the N-terminus of 6His-VP55, its interaction with the 6His-VP39 surface was detected (Fig. 4A). A relatively small amount of VP55 interacted because of its dilution during tag cleavage and desalting, and an apparently low specific binding activity of *E. coli*-expressed VP39 with respect to the vaccinia-expressed VP39 used in all other experiments. The immobilization of two proteins that were C-terminally tagged with 6His was also attempted, viz. VP39, and human La protein - a putative RNA polymerase III transcription termination factor (Gottlieb and Steitz, 1989; Gottlieb and Steitz, 1989; Meerovitch et al., 1993; Maraia et al., 1994). Each interacted stably with the NTA-derivatized flow cell (Fig. 4B, C). The identities of the respective immobilized species were confirmed in additional experiments, by the injection of VP55 and an anti-La protein monoclonal antibody (data not shown).

3.5. Complete regeneration of NTA-derivatized surfaces

Routinely, greater than 99% of immobilized protein could be eluted from NTA-derivatized surfaces under standard elution conditions (0.5 M EDTA, pH 8.0). However, the remaining material could accumulate over many cycles of protein binding and EDTA elution. This eventually led to promiscuous and non-authentic Ni^{2+} and protein interactions with the non-regenerable protein, presumably due to its gradual conformational rearrangement. Therefore, NTA-derivatized surfaces were purged every 50–100 cycles with a 10 min injection of proteinase K (1 μg/ml) in 2% SDS, followed by 2% SDS then 0.5 M EDTA. This procedure completely and consistently regenerated the immobilized ligand, and did not adversely affect subsequently injected proteins. With periodic purging, NTA-derivatized flow cells have retained chelating activity over months of intermittent use and storage, and hundreds of binding-elution cycles. Overall lifetimes of NTA-derivatized surfaces have not been determined, since an irreversible loss of chelating activity has not been observed.

3.6. Utility of oligohistidine-tagged protein immobilization via a chelating linkage

We have described the stable immobilization of four recombinant proteins, tagged at either terminus with oligohistidine, using a chelating

linkage. Although, to our knowledge, NTA ligand is not commercially available, the procedure for its synthesis from commercially available reagents is relatively straightforward (Hochuli et al., 1987). The chelating immobilization method may be generally applicable with oligohistidine-tagged recombinant proteins. Although we did not explore the selective immobilization of oligohistidine tagged proteins directly from crude extracts, such schemes may also be feasible. The possible advantages of the chelating linkage include an increased 'availability' of immobilized molecules, as shown above for 6His-VP55. In addition, the procedure may be useful for proteins that are incompatible with the low pH conditions often employed for the 'pre-concentration' step of covalent coupling via primary amino groups. A further advantage might be the initiation of each interaction cycle with the immobilization of fresh 6His-tagged protein, and its termination with protein elution. This not only facilitates experiments requiring frequent optimization or adjustment of immobilized protein levels by facilitating the multiple re-use of expensive biosensor chips, but also minimizes the time available for immobilized protein to lose activity.

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